Oxidation and Nitrosylation of Cysteines Proximal to the Intermediate Filament (IF)-binding Site of Plectin

EFFECTS ON STRUCTURE AND VIMENTIN BINDING AND INVOLVEMENT IN IF COLLAPSE*S

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plectin plays a key role in the maintenance of cellular cytoarchitecture and serves at the same time as a scaffolding platform for signaling cascades. Consisting of six structural repeats (R1-6) and harboring binding sites for different IF proteins and proteins involved in signaling, the plectin C-terminal domain is of strategic functional importance. Depending on the species, it contains at least 13 cysteines, 4 of which reside in the R5 domain. To investigate the structural and biological functions of R5 cysteines, we used cysteine-to-serine mutagenesis and spectroscopic, biochemical, and functional analyses. Urea-induced unfolding experiments indicated that wild-type R5 in the oxidized, disulfide bond-mediated conformation was more stable than its cysteine-free mutant derivative. The binding affinity of R5 for vimentin was significantly higher, however, when the protein was in the reduced, more relaxed conformation. Of the four R5 cysteines, one (Cys4) was particularly reactive as reflected by its ability to form disulfide bridges with R5 Cys1 and to serve as a target for nitrosylation in vitro. Using immortalized endothelial cell cultures from mice, we show that endogenous plectin is nitrosylated in vivo, and we found that NO donorinduced IF collapse proceeds dramatically faster in plectin-deficient compared with wild-type cells. Our data suggest an antagonistic role of plectin in nitrosylation (oxidative stress)mediated alterations of IF cytoarchitecture and a possible role of R5 Cys4 as a regulatory switch.

As an intermediate filament (IF)-based cytolinker protein,

Plectin, a cytoskeletal linker protein of very large size, is widely expressed in mammalian tissues and different cell types. It interlinks intermediate filaments (IFs),³ connects them to

actin and microtubule networks, and anchors them to the subplasma membrane skeleton and to plasma membrane-cytoskeleton junctional complexes (reviewed in Ref. 1). However, the cellular functions of plectin are not merely that of a structural linker. Emerging evidence indicates that plectin also serves as a scaffolding platform in the assembly of signaling complexes (2-5), with a potential of promoting finely tuned protein interactions that ultimate govern the spatiotemporal regulation of cell migration. Previous biochemical and immunolocalization studies led to the concept that plectin plays an important structural role in maintaining the mechanical stability of cells. This concept was fully confirmed when epidermolysis bullosa simplex-muscular dystrophy, a severe skin blistering disease combined with muscular dystrophy, was linked to mutations in the plectin gene (6). A similar phenotype was found in plectin knock-out mice, which exhibit severe skin blistering within the basal layer of the epidermis, reduced numbers of hemidesmosomes, and abnormalities in heart and skeletal muscle (7).

Plectin belongs to the plakin family of cytolinker proteins, which also includes desmoplakin, BPAG1, ACF7/microtubule actin cross-linking factor, envoplakin, periplakin, and epiplakin (8). Like most of the other proteins in this family, plectin has a multidomain structure consisting of a globular N-terminal domain; a central rod domain, and a globular C-terminal domain, which itself is multimodular. Electron microscopy of purified plectin molecules confirmed their three-domain structure (9). Functionally, the N-terminal domain harbors an actinbinding domain; the central rod domain mediates dimerization; and the C-terminal domain contains the IF-binding site. The building block of the plectin C terminus is a repeat (R) domain known as the plakin or plectin repeat domain (8, 10). R domains consist of a conserved core domain or module built from 4.5 tandemly repeated copies of a 38-amino acid motif. The modules are separated from each other by linker sequences of variable lengths. The concept of the module as the basic structure of the C-terminal domain of plakin proteins has been validated when the crystal structure of two desmoplakin R domains was solved (11). The C terminus of plectin (~1900 amino acids) features six highly homologous R domains (R1-6) of \sim 300 amino acids each. Accommodation of the six R domains of plectin within its globular C-terminal domain, which has an estimated diameter of 9 nm (9), requires tight packing. Janda et al. (10) proposed a circular arrangement of antiparallel-oriented plectin R1-5 domains with the R6 domain in their center;

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The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures," Equations 1 and 2, Refs. 1–6, and Figs. S1 and S2.

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³ The abbreviations used are: IFs, intermediate filaments; R, repeat; DTT, dithiothreitol; BSA, bovine serum albumin; SNAP, S-nitroso-N-acetylpenicillamine; NOS, nitric-oxide synthase; PMA, phorbol 12-myristate 13-acetate.

such a structure could be stabilized by hydrophobic interactions between residues on the surface of each R domain and/or through cysteines via disulfide bond formation due to physiological bursts of oxidative stress.

The C-terminal part of plectin contains from 13 (mouse) to 17 (human) cysteines (12, 13). Four of these generally highly conserved residues are clustered in the plectin R5 domain, which harbors also the major IF-binding site of the protein (14). This provocative localization might serve a functional purpose, as these cysteines could form intra- and inter-repeat disulfide bridges, providing more structural rigidity to the protein itself. In addition, they may mediate interactions of the protein with various binding partners with docking sites in this region, such as various IF subunit proteins (reviewed in Ref. 1) and proteins involved in cellular signaling, including RACK1 (3), a regulator of protein kinase C, and AMP-activated protein kinase (4), a key regulatory enzyme of energy homeostasis. Intracellular disulfide bridge formation is likely to be of particular importance in situations in which cells have to respond to mechanical, oxidative, or other types of stress. Because full-length plectin or even entire subdomains, such as the C-terminal globular domain with its six R domains (>500 and >200 kDa, respectively), are too large to be recombinantly expressed for biochemical analyses, this study was restricted to the plectin R5 domain.

The following questions regarding R5 cysteines were addressed. Does their oxidation change the biochemical and conformational properties of the protein? Are they accessible to disulfide cross-linking within R5 and between R5 and R4? Is the binding affinity of R5 for vimentin affected by their oxidative state? Are they targets of nitrosylation, and if so, does nitrosylation occur *in vivo*, and does it have consequences for plectin-regulated cellular functions?

EXPERIMENTAL PROCEDURES

Sequences of Constructs, Plasmids, and Site-directed Mutagenesis—The amino acid sequences of the plectin R4 and R5 domains consist of residues 3780–4024 and 4025–4367 (Swiss-Prot accession number P30427), respectively. R4 and R5 were amplified by PCR using a mouse plectin cDNA comprising exons 31 and 32 (12) as template and subcloned into pBN120, a pET15b derivative (14). Mutagenesis was carried out by an overlap extension approach or using the QuikChange site-directed mutagenesis kit (Stratagene) with primers designed to substitute cysteines with serines. All constructs were verified by automated DNA sequencing. For transfection of cultured cells, selected cDNA constructs were subcloned into pAD29, an SV40 promotor-driven expression vector containing a C-terminal Myc tag (14). The bacterial expression plasmid pFS129 was the source of mouse full-length vimentin (15).

Expression and Purification of Proteins—Recombinant proteins were expressed in *Escherichia coli* strain BL21(DE3) after induction with 1 mM isopropyl β -D-thiogalactopyranoside. Unless otherwise indicated, His-tagged recombinant proteins were prepared under reducing conditions and affinity-purified on nickel columns (His·Bind, Novagen) following the protocol of the manufacturer. The bacterial cell pellets were resuspended in a lysis buffer containing 10 mM piperazine (pH 11.0), 1% Triton X-100, and 10 mM β -mercaptoethanol, and the binding, washing, and elution column buffers (20 mM Tris-HCl (pH 9.0) containing 5, 20, and 250 mM imidazole, respectively) were supplemented with 5 mM β -mercaptoethanol. Proteins were kept in elution buffer at 4 °C and dialyzed against the required buffer prior to being assayed. All solutions were degassed and supplemented with β -mercaptoethanol until the dialysis step, except in europium binding assays and CD spectroscopy, in which no β -mercaptoethanol was added. Recombinant mouse full-length vimentin was purified by sequential ion-exchange column chromatography on DEAE-Sepharose and CM-Sepharose (GE Healthcare) as described previously (15). Also in this case, all buffers were degassed. Protein concentrations were estimated by the Bradford or BCA (Pierce) method.

SDS-PAGE and Immunoblotting—Protein samples (10 μ g/20 μ l) were analyzed by SDS-10% PAGE under reducing and nonreducing conditions. The reduction of samples before application to the gel was achieved by the addition of 0.2 M dithiothreitol (DTT) to 2× sample buffer. After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue, 45% methanol, and 10% acetic acid and scanned in an HP ScanJet 8250 scanner. Densitometric analysis was performed with Gel Doc 2000 gel documentation system (Bio-Rad) and Quantity One image analysis software (Bio-Rad). Transfer to 0.2- μ m nitrocellulose membranes (Protran[®], Schleicher & Schüll) and immunoblotting were done following standard procedures.

Spectroscopy and Urea-induced Unfolding—The details of far-UV CD and fluorescence emission spectroscopy, including sample preparation, are provided in supplemental "Experimental Procedures."

Oxidative Cross-linking—Purified recombinant proteins at a concentration of 0.5 mg/ml were dialyzed overnight at 4 °C against 10 mM Tris-HCl (pH 7.9) while being concurrently exposed to oxidation by air (no precautions were taken to exclude air from the solutions). The reaction was quenched by the addition of iodoacetamide (final concentration of 50 mM) to block free sulfhydryl groups. Samples were then resolved by SDS-10% PAGE under nonreducing or reducing conditions. Alternatively, aliquots of two different recombinant proteins were mixed 1:1 at a concentration of 0.5 mg/ml each; dialyzed against 10 mM Tris-HCl (pH 7.9), 6 M urea, and 1 mM DTT for 1.5 h at room temperature; and subsequently oxidized by air while being dialyzed at 4 °C into 10 mM Tris-HCl (pH 7.9) without urea. The reactions were then quenched and analyzed as described above.

Blot Overlay Binding Assay—Purified proteins (0.5 μ g/lane) were subjected to SDS-10% PAGE and blotted onto nitrocellulose membranes. The membranes were blocked for 1 h with 3% bovine serum albumin (BSA) in phosphate-buffered saline; incubated for 1 h with purified samples of vimentin (10 μ g/ml) in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, 1 mM EGTA, 2 mM MgCl₂, 1 mM DTT, and 0.1% (v/v) Tween 20 (pH 7.5); and washed several times with phosphatebuffered saline containing 0.05% (v/v) Tween 20. Bound vimentin was detected using affinity-purified goat anti-mouse antibodies to vimentin (diluted 1:5000; kindly provided by Peter Traub, University of Bonn, Bonn, Germany) (16), followed by incubation with horseradish peroxidase-conjugated anti-goat IgG (diluted 1:5000; Jackson ImmunoResearch Laboratories,



Inc.). Immune complexes were detected by chemiluminescence (West Pico ECL, Pierce).

Co-sedimentation Assay—Co-sedimentation assays were carried out essentially as described by Choi *et al.* (11) with minor modifications regarding protein preparation (see above) and buffer composition. To prepare vimentin filaments, recombinant vimentin was dialyzed stepwise at room temperature against 5 mM Tris acetate (pH 8.3), 1 mM EDTA, and 10 mM β -mercaptoethanol with decreasing concentrations of urea (6 to 0 M). The dialyzed vimentin solution was centrifuged in a Beckman benchtop ultracentrifuge (OptimaTM TLX) at 100,000 × g for 30 min at room temperature, and soluble vimentin (8.9 μ M) was polymerized in 10 μ l of 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.1% (v/v) Tween 20 (buffer A) for 30 min at 37 °C.

Recombinant plectin R5 domains (wild-type and cysteinefree mutant) were heated in elution buffer supplemented with 0.1% (v/v) Tween 20 for 10 min at 37 °C and dialyzed sequentially against 20 mM Tris-HCl (pH 8.1) and 0.1% (v/v) Tween 20 (buffer B), buffer B supplemented with 1 mM EDTA, and buffer A. All solutions were degassed. Recombinant R4 was dialyzed against the same set of buffers without an initial heating step. R4 and wild-type and cysteine-free R5 were then added to polymerized vimentin (8.9 μ M in 10 μ l of buffer A) at different concentrations (1–10:1 molar ratios of vimentin to plectin). After the addition of buffer A to a total volume of $50-100 \mu$ l, the mixtures were incubated for 1 h at 37 °C. Vimentin filaments and associated protein were pelleted by centrifugation at $100,000 \times g$ for 30 min at room temperature using the Beckman OptimaTM TLX ultracentrifuge. Supernatants were withdrawn immediately after centrifugation, and an equal volume of $2\times$ SDS-PAGE sample buffer was added. Pellets were dissolved in 50 μ l of 2× SDS-PAGE sample buffer and 50 μ l of buffer A. The supernatant and pellet fractions were analyzed by SDS-10% PAGE. For controls without vimentin, 8.9 µM R4, wild-type and cysteine-free R5, and BSA were treated in the same manner.

Europium Binding Assay—Recombinant vimentin dialyzed stepwise against 50 mM NaHCO₃ (pH 8.5) and labeled with Eu³⁺ was overlaid in serial dilutions onto 96-microtiter plates coated with wild-type and cysteine-free R5 (both at 100 nM). Bound protein was determined by releasing the vimentinbound Eu³⁺ with enhancement solution and measuring fluorescence with a Wallac time-resolved microtiter plate fluorometer (excitation wavelength of 340 nm and emission wavelength of 615 nm). Further details of this assay have been described previously (14, 15). The Scatchard method was used for analysis of binding data, and fluorescence values were converted to concentrations by comparison with an Eu³⁺ standard.

Cell Culture and Immunofluorescence Microscopy—The details of the procedures used for the isolation and immortalization of endothelial cells from mouse kidneys and of cell processing for immunofluorescence microscopy are given under supplemental "Experimental Procedures." Endothelial cells to be analyzed by immunofluorescence microscopy after NO donor-mediated nitrosylation were grown on gelatin-coated glass coverslips; treated with 100 μ M *S*-nitroso-*N*-acetylpenicillamine (SNAP) in the dark for 2, 4, or 6 h; washed with phosphate-buffered saline; and then fixed.

Cysteines Proximal to the Plectin IF-binding Site

S-Nitrosylation Assays-S-Nitrosylated proteins were detected using the biotin-switch assay as described by Jaffrey and Snyder (17). For in vitro assays, purified wild-type R5 and R5-C4S (80 µg each) in 250 mM HEPES (pH 7.7), 1 mM EDTA, and 0.1 mM neocuproine (HEN buffer) were incubated in the dark with 100 μ M SNAP for 2 h at room temperature, and the NO donors were then removed by passing the samples twice through a desalting column (Micro Bio-Spin P-6, Bio-Rad). Proteins in the flow-through were blocked with 20 mM methyl methanethiosulfonate (Sigma) for 20 min at 50 °C, precipitated with acetone for 20 min at -20 °C, and collected by centrifugation at 10,000 \times g for 10 min at 4 °C. Pellets were resuspended in 500 μ l of HEN buffer containing 1% SDS (HENS buffer) and incubated with 1 mm ascorbic acid to release NO from thiol groups, which were subsequently biotinylated with 1 mM N-(6-(biotinamido)hexyl)-3'-(2'-pyridyldithio)propionamide (Pierce). Proteins were again acetone-precipitated and resuspended in 300 µl of HENS buffer, and biotinylated proteins were recovered by streptavidin affinity chromatography. Eluted proteins were separated by SDS-10% PAGE; transferred to nitrocellulose membranes; analyzed by immunoblotting using antibodies to plectin (diluted 1:2500; No. 9) (18), endothelial nitric-oxide synthase (NOS; diluted 1:1000; BD Transduction Laboratories), and His tag (diluted 1:1000; Qiagen Inc.); and visualized by chemiluminescence. For the identification of S-nitrosylated proteins in cultured cells, confluent mouse renal endothelial cells ($\sim 2.5 \times 10^7$) were incubated with 100 μ M SNAP for 2 h in the dark or with 100 nm phorbol 12-myristate 13-acetate (PMA) for 20 h, washed thoroughly with phosphatebuffered saline, scraped off, lysed in 350 μ l of HEN buffer supplemented with 2.5% SDS, blocked with methyl methanethiosulfonate, and subjected to the biotin-switch assay as described above.

Homology Modeling-A model was generated using an automated homology modeling server (ExPASy proteomics server using SWISS-MODEL ProModII) running at the Swiss Institute of Bioinformatics (Geneva, Switzerland) and Geno3D (Lyon, France). The structural template used for modeling was the crystal structure of human desmoplakin repeat domain B (Protein Data Bank code 1LM7) (11), which shares 73% sequence identity with the plectin R5 module. Modeling by satisfaction of spatial restraints was performed by the method of Combet *et al.* (19). In brief, the alignment of the target (plectin R5 module) and the template (desmoplakin domain B) was obtained using the Needleman-Wunsch global alignment algorithm on the EMBL-EBI server (www.ebi.ac.uk/emboss/align/). Restraints on various distances, angles, and dihedral angles in the sequence were derived from the alignment of the target with the template structure. Finally, the three-dimensional model was refined by applying distance geometry, simulated annealing, and energy minimization procedures. Visualization of the three-dimensional structure was performed using the molecular graphics package Yasara (www.yasara.com).

RESULTS

Predicted Three-dimensional Structure of the Plectin R5 Domain and Localization of Cysteines—The C-terminal R domains of plakin protein family members have been classified







FIGURE 1. **Proposed structural model of plectin R5.** *A*, sequence alignment of the template (desmoplakin domain B) and the plectin R5 domain with secondary structure assignments derived from the homology model. *Arrows* and *ribbons* indicate β -strands and α -helices, respectively. Residues shown in *red*, *green*, or *black* are identical, similar, or different, respectively. Cysteine residues are marked by *asterisks*. *B*, homology model of the plectin R5 module based on the structure of desmoplakin repeat domain B, represented as a *ribbon diagram*. PLEC repeats 1–5 are highlighted in *red*, *yellow*, *blue*, *orange*, and *green*, respectively. The PLEC fold is characterized by two antiparallel β -sheets that form a β -hairpin and two antiparallel α -helices. Cysteine residues (Cys1–3) are numbered sequentially according to their position in the polypeptide chain. *C*, sequence alignment of the linker region connecting repeat domains B and C of desmoplakin and R5 and R6 of plectin. The positions of Cys4 (*) and the unique tryptophan (#) are indicated. The nitrosylation consensus sequence is *boxed*. *D*, schematic representation of the wild-type (wt) R5 domain and the mutant versions used in this study. Only cysteine residues (*open ovals*) and their serines replacements (*filled ovals*) are shown. Cys1–4 of R5 correspond to residues 4074, 4248, 4257, and 4316 in full-length plectin (Swiss-Prot accession number P30427).

as type A, B, and C (20). Desmoplakin has three such domains, one of type A, one of type B, and one of type C, whereas of the six plectin R domains, five are type B (R1–5), and one is type C (R6) (21). Within the repeat regions, plectin and desmoplakin share a high sequence similarity, and all but one of the cysteines present in the plectin R1–6 domains are present in desmoplakin R domains A–C.

To gain insight into the location and accessibility of the cysteine residues within R5, we generated, using an automated homology modeling server (SWISS-MODEL and Geno3D), a three-dimensional model of the plectin R5 module based on the recently solved crystal structure of desmoplakin R domain B (Protein Data Bank 1LM7) (11). The alignment of the target (plectin R5 module) and the template (desmoplakin domain B), obtained using the Needleman-Wunsch global alignment algorithm on EMBL-EBI server, is shown in Fig. 1*A*. The overall topology of the plectin R5 three-dimensional structure (Fig. 1*B*) was found to be similar to that of the model template, yet distinct small differences are likely to confer unique properties to plectin. Like desmoplakin R domain B, plectin R5 comprises five homologous 38-amino acid-long structural subunits called the PLEC repeat (SMART accession number SM00250). Each of the five PLEC repeats adopts a fold that, similar to the one described for the plakin repeat of desmoplakin, is dominated by two antiparallel β -sheets (forming a β -hairpin) and two antiparallel α -helices (Fig. 1, *A* and *B*). The fold is remarkably similar to that of the ankyrin repeat, as it was predicted by threading analysis (10). Conserved hydrophobic residues in the β -hairpin contribute to the packing within each PLEC repeat and promote the adoption of a globular, cylinder-like structure that is

The Journal of Biological Chemistry

45 Å long with a 25-Å diameter. Cys1 is located in the β -hairpin of the first PLEC repeat, whereas Cys2 and Cys3 are in the terminal part of the fifth PLEC repeat, which interacts with the first PLEC repeat (Fig. 1B). Cys2 and Cys3 are on the surface of the structure, whereas Cys1 is partially buried in a groove. The crystallized desmoplakin fragment does not include the linker region containing the corresponding Cys4. There is evidence, however, that this cysteine, residing in the loop connecting R domains B and C, is exposed, as partial chymotryptic digestion of the bacterially expressed C-terminal domain of desmoplakin results in cleavage of the polypeptide within this R domain linker region (11). Secondary structure predictions for this region suggest that Cys4 is located in a short unstructured sequence connecting an α -helical segment and a β -sheet (Fig. 1A). This linker region has also one of the highest sequence conservations among different plakin family members (22). An alignment of the corresponding linker regions of plectin and desmoplakin is shown in Fig. 1C.

Expression of Functional Wild-type and Mutant R5 Domains-To assess the potential of individual R5 cysteines to form disulfide bridges, we generated recombinant versions of R5 in which all four cysteines were replaced with serines either individually or in different combinations. Cys1 and Cys4 were mutated alone and in various combinations with Cys2 and Cys3, including a mutant without any cysteine (Cys-free R5). In addition, we generated a mutant with native Cys1 and Cys4, but without Cys2 and Cys3 (R5-C2S,C3S), and its counterpiece with Cys2 and Cys3, but without Cys1 and Cys4 (R5-C1S,C4S). Schematics of wild-type R5 and the eight mutant versions generated and their assigned names are shown in Fig. 1D. The wild-type and mutant proteins were expressed to a similar extent and were equally well soluble, except for the cysteinefree mutant, which showed a slight tendency to aggregate. Proteins were purified to homogeneity, and their purity and functional competence were verified by SDS-PAGE and co-sedimentation with in vitro assembled vimentin IFs (data not shown).

The overexpression of R5 leads to the collapse of IF networks in different types of cells (14, 15). To further demonstrate that the mutations had not caused significant changes in R5 function, we transfected mammalian expression plasmids encoding Myc-tagged versions of wild-type and Cys-free R5 domains into PtK2 and mouse fibroblast cells. As monitored by immunofluorescence microscopy, overexpression of either protein led to IF collapse in both cell types after 2 days (supplemental Figs. S1 and S2), without any detectable phenotypic differences. Similarly, when immortalized keratinocytes (18) were subjected to this type of analysis, in agreement with previous results (14), a decoration but not a collapse of keratin networks was observed with both versions of R5 (data not shown). There was also no detectable difference between the two R5 variants regarding effects on actin filament or focal adhesion contact organization in fibroblasts (data not shown). Thus, the specific replacement of cysteines with serines did not appear to alter the basic association patterns of plectin with its cellular partners, strongly suggesting that the mutants were functional.

Cysteines Proximal to the Plectin IF-binding Site

Biochemical and Conformational Properties of Wild-type R5 Compared with Its Cysteine-free Variant—Optical spectroscopic methods were used to reveal structural differences and changes in thermodynamic stability associated with the elimination of all four cysteines in R5. In particular, far-UV CD spectroscopy was used to obtain global information about the secondary structure content, whereas the intrinsic fluorescence of the single tryptophan located in close vicinity of Cys4 (Fig. 1*C*) was exploited as a local probe of the tertiary structure.

The far-UV CD spectrum of wild-type R5 (Fig. 2A, upper panel) measured at pH 7.5 showed two minima at 222 and 208 nm of almost equal negative ellipticity, typical for a high content of α -helical structure. In contrast, the second minimum in the spectrum of cysteine-free R5 (Fig. 2A, upper panel) not only was shifted toward 205 nm, but showed an increased negative ellipticity, indicating a higher content of random coil structure. These findings were verified by estimation of the secondary structure content using three different algorithms (SELCON, CONTIN, and CDSSTR), which yielded consistently 22% α -helical, 27% β -sheet, and 50% random coil content for wildtype R5, whereas for cysteine-free R5, the α -helical content was reduced to 17% in conjunction with an increased random coil content of 55%. Most important, the far-UV CD spectra of cysteine-free R5 recorded at pH 9 and 11 (Fig. 2A, lower panel) became essentially identical to those of wild-type R5, which were pH-insensitive (middle panel). In sum, the CD spectra of the wild-type and cysteine-free mutant R5 proteins were essentially identical, indicating that the substitution had a negligible effect on the overall secondary structure and did not cause large structural perturbation. Additionally, it can be concluded that the contribution of the four R5 cysteine residues to the far-UV CD signal is negligible.

The fluorescence spectrum of the single tryptophan at pH 7.5 exhibited an emission maximum at 335 nm for wild-type R5, whereas for the cysteine-free mutant, the maximum was shifted to 341 nm (data not shown), indicating a less buried conformation of the environment-sensitive tryptophan residue (23). Incubation of both proteins in 8 M urea resulted in red shifts to 348.5 nm for the wild-type protein and to 350.5 nm for the mutant protein, indicating, in both cases, an exposure of the tryptophan residue to the polar solvent. SDS-PAGE of samples of wild-type R5 in its native state (no urea) showed that the vast majority of wild-type R5 used for the measurement was in the oxidized monomeric form (data not shown), resulting from disulfide bond formation between Cys1 and Cys4 (see below). As a consequence, the tryptophan residue neighboring Cys4 became buried within the protein structure.

The thermodynamic stability was determined by urea-induced unfolding at pH 7.5 monitored by far-UV CD and tryptophan fluorescence (Fig. 2*B*). The unfolding of wild-type R5 showed sigmoidal transition curves for both far-UV CD and fluorescence with transition midpoints at 3.1 and 2.9 M urea, respectively. In contrast, the unfolding profiles for the cysteinefree mutant were clearly not coincident. The transition midpoint of the tryptophan fluorescence signal was at 1.5 M urea, whereas the far-UV CD unfolding profile reflected a very broad transition without a distinct base line for the native state. This behavior indicates the existence of intermediate states, where



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FIGURE 2. Structural analysis of wild-type and cysteine-free R5. *A*, far-UV CD spectra and secondary structure contents at various pH values. *Upper panel*, wild-type (\bigcirc) and cysteine-free (\bigcirc) R5 in 10 mm sodium phosphate buffer (pH 7.5) and 20 mm NaF at room temperature; *middle panel*, wild-type

the tryptophan fluorescence reports only the unfolding of the C-terminal part of R5. Free energies of unfolding were not calculated, as these calculations require the proteins to follow a two-state transition. However, the observed transition midpoints allowed a semiquantitative comparison of the thermodynamic stabilities of wild-type R5 and the cysteine-free mutant.

Disulfide Cross-linking within and between Plectin R5 and R4—To identify cysteine residues within R5 with reactive thiol groups that could engage in disulfide bridge formation, R5 and the complete mutant collection were tested for changes in their electrophoretic mobility due to formation of disulfide-linked dimers or higher oligomers. For this purpose, purified recombinant proteins were oxidatively cross-linked by exposure to air at 4 °C to promote disulfide bond formation, subsequently treated with iodoacetamide to block free cysteine residues, and then subjected to SDS-PAGE in the presence or absence of the reducing agent DTT. In the presence of DTT, wild-type R5 and all mutant proteins occurred in the monomeric form characteristic of wild-type R5, migrating with an apparent molecular mass of 41 kDa (Fig. 3A, lane 10) (data not shown). In contrast, in the absence of DTT, several proteins bands corresponding to monomeric, dimeric, and higher order oligomeric forms were observed (Fig. 3A, lanes 1-9). Wild-type R5 occurred as a reduced (~41 kDa) and oxidized (~38 kDa) monomer and was able to form dimers, trimers, tetramers, and other oligomers (Fig. 3A, lane 1). The faster migration of the oxidized form compared with the reduced form of the monomer is typical for intramolecular disulfide bonding, resulting in a more tightly folded structure (24, 25). R5-C2S,C3S gave a cross-linking pattern similar to that of wild-type R5 (Fig. 3A, lane 8). Because the only cysteines available in the R5-C2S,C3S mutant are Cys1 and Cys4, we concluded that the oxidized forms of the monomer were presumably formed by disulfide bond formation between these two residues. Mutants R5-C1S and R5-C1S,C2S,C3S displayed two prominent bands corresponding to the reduced form of the monomer (\sim 41 kDa) and dimers with an abnormal mobility of \sim 100 kDa (Fig. 3*A*, *lanes 2* and 5). We believe that these dimers were formed by disulfide pairing of two Cys4 residues in two different R5 molecules. Cys4 resides in the linker region between modules 5 and 6 at a relatively large distance from the core region of R5. Disulfide bond formation between two Cys4 residues may therefore generate dimers with a larger hydrodynamic dimension, thus migrating slower (apparent molecular mass of ~100 kDa instead of ~82 kDa) upon SDS-PAGE due to their extended conformation (26, 27). In contrast, the cross-linked R5-C4S mutant formed a dimer migrating at

R5 at pH 7.5 (•), pH 9.0 (•), and pH 11.0 (•); *lower panel*, cysteine-free R5 at pH 7.5 (•), pH 9.0 (•), and pH 11.0 (•). *B*, urea-induced unfolding profiles monitored by far-UV CD and fluorescence spectroscopy. The fraction of unfolded protein observed by far-UV CD and fluorescence spectroscopy is plotted against the concentration of urea. *Symbols* represent the spectroscopic signals obtained, and *solid lines* represent the profiles obtained on the basis of Equations 1 and 2 as described under supplemental "Experimental Procedures." *Closed symbols* indicate normalized changes in ellipticity at 222 nm (•) and fluorescence emission at 340 nm after excitation at 290 nm (•) for wild-type R5. *Open symbols* (2.8 μ M) were in 10 mM sodium phosphate buffer (pH 7.5). *qMRW*, mean residue weight ellipticity; *deg*, degrees.



FIGURE 3. **Disulfide cross-linking within and between plectin R5 and R4.** *A*, analysis of cross-linked products formed by wild-type (*wt*) and mutant R5. Proteins oxidized by air were subjected to SDS-10% PAGE under nonreducing (*lanes* 1–9) or reducing (*r*; *lane* 10) conditions. For reducing conditions, 0.2 M DTT was added to $2\times$ sample buffer to achieve a final concentration of 0.1 m. *B*, cross-linking of R4 with wild-type and cysteine-free R5. *Lanes* 1–3, cross-linked products of the indicated single R domains. Conditions were similar to *A* except that 6 m urea was added to the sample buffer. *Lanes* 4 and 5, cross-linked products of two repeat domains. R4 was mixed with either wild-type or cysteine-free R5 and air-oxidized. Samples were resolved by SDS-10% PAGE under nonreducing conditions. The molecular masses of monomers and dimers of R4 are \sim 30 and \sim 60 kDa, and those of R5 are \sim 38, \sim 41, and \sim 82 kDa (see "Results" for discussion). The *arrow* indicates the heterodimer band (\sim 66 kDa) formed between R4 and wild-type R5. *Lanes* M, molecular masses.

the expected mobility of ~82 kDa (Fig. 3*A*, *lane 7*). Cys-free R5 occurred only as the reduced form of the monomer (~41 kDa) (Fig. 3*A*, *lane 6*). In addition to a protein band with an apparent molecular mass of ~100 kDa, mutant R5-C1S,C3S yielded a band corresponding to a higher oligomer (Fig. 3*A*, *lane 3*). The rest of the mutants, R5-C1S,C2S, and R5-C1S,C4S, remained in the monomeric form after oxidation (~41 kDa) (Fig. 3*A*, *lanes 4* and *9*).

These results provided evidence for the formation of an intra-repeat domain disulfide bridge between Cys1 and Cys4. When either Cys1 or Cys4 was not available, oxidative conditions promoted inter-repeat cross-linking (i.e. between distinct R5 domain molecules) and yielded two different types of dimers depending on the cysteine residue engaged in the disulfide bridge. Inter-repeat domain cross-linking via Cys1 delivered dimers in the regular conformation, migrating with the expected apparent molecular mass of 82 kDa (Fig. 3A, lane 7), whereas inter-repeat domain cross-linking via Cys4 delivered dimers with an extended conformation that migrated slower. Bridges between the other cysteines were unlikely to have occurred because of distance restrictions between the cysteine residues. (The distance between the sulfhydryl groups of Cys2 and Cys3 (5.691 Å) is beyond the optimum of 2.4 Å for creating a disulfide bridge.) This assumption is supported by the fact that, in the crystal structure of domain B of desmoplakin (obtained in the absence of reducing agents) (11), disulfide bonding between Cys²²⁵⁹, Cys²⁴³³, and Cys²⁴⁴², which are equivalent to Cys1, Cys2, and Cys3, was not observed. Because the available high resolution structural data on desmoplakin do not include the region where Cys4 resides, from structural

reducing conditions for 1.5 h in the presence of 6 M urea, followed by dialysis to remove urea and by SDS-PAGE, both without reducing agents. Because of the size difference of R4 and R5 (\sim 30 and \sim 41 kDa, respectively), a band of a size between that of the homodimers (\sim 61 and \sim 82 kDa, respectively) would be indicative of heterodimer formation. Such a band was indeed observed (Fig. 3*B*, *lane* 4). This band was absent when R4 was incubated with the cysteine-free R5 mutant (Fig. 3*B*, *lane* 5). Thus, the single cysteine in R4 as well as cysteines in R5 formed disulfide bonds not only between their own molecular entities (R4/R4 and R5/R5 homodimers), but also between each other (R4/R5 heterodimers).

Increased Vimentin Binding Affinity of Plectin R5 in the Reduced Compared with the Oxidized Form-The major vimentin-binding site of plectin is located in the linker region connecting the R5 and R6 domains, where also Cys4 is found (Fig. 1C). It was therefore of special interest to examine whether this cysteine plays any role in vimentin-R5 interaction. In a first series of experiments, vimentin binding was assessed by cosedimentation of wild-type or cysteine-free R5 with IFs assembled from recombinant vimentin in vitro. After incubation of mixtures containing vimentin and R5 proteins at different molar ratios (1:1, 5:1, and 10:1), filaments were sedimented, and supernatants and pellets were analyzed by SDS-PAGE, followed by densitometric quantification of protein bands. Samples containing R4 or BSA (at 1:1 molar ratios to vimentin) instead of R5 and additional controls containing just vimentin, BSA, R4, or R5 (without the corresponding binding partner) were processed and analyzed under similar conditions. At a vimentin/R5 ratio of 1:1, nearly 90% of R5 co-sedimented with vimentin,

We speculated earlier that plectin R domains may bind to each other not only because of hydrophobic and electrostatic interactions, but also because of disulfide bond formation between the repeats (10). To test this hypothesis, we first examined whether the single cysteine residue present in R4 is able to form a disulfide bond between two R4 molecules. When R4 oxidatively crosslinked under nonreducing conditions was analyzed, two bands were found, one corresponding to the monomer and the other to the dimer, both with the expected mobilities (Fig. 3B, lane 1), whereas a single monomeric band was observed under reducing conditions (data not shown). Next, we tested whether R4 and R5 could be linked via a disulfide bridge. Heterodimer formation was examined by incubating R4 with wild-type R5 under



FIGURE 4. Vimentin binding of plectin domains. A, co-sedimentation of plectin R domains with vimentin filaments. Polymerized vimentin (Vim) was incubated with plectin R domains (RDs) at the following final concentrations: 8.9 μM vimentin and 8.9 μM R domain (1:1), 8.9 μM vimentin and 1.78 μM R domain (5:1), and 8.9 μM vimentin and 0.89 µM R domain (10:1). After centrifugation, supernatant (S) and pellet (P) fractions were analyzed by SDS-10% PAGE. Lanes 1, vimentin + BSA; lanes 2, vimentin + R4; lanes 3, 5, and 7, vimentin + wild-type (wt) R5; lanes 4, 6, and 8, vimentin + Cys-free R5. B, analysis of pellets obtained after incubation of vimentin, BSA, wild-type R5, Cys-free R5, and R4 alone at a concentration of 8.9 μ M in buffer A. The Position of vimentin is indicated ([dharrow]). C, overlay of plectin R domains with vimentin. Left panel, Coomassie Blue staining of recombinant proteins fractionated by SDS-10% PAGE; right panel, a gel similar to that shown in the left panel transblotted onto a nitrocellulose membrane and overlaid with recombinant mouse full-length vimentin (10 μ g/ml). For detection of bound vimentin, the membrane was then probed with a monoclonal antibody to vimentin, followed by horseradish peroxidase-conjugated secondary antibodies, and developed by ECL using the manufacturer's protocol. *Lane M*, molecular mass markers. *nr*, nonreduced; *r*, reduced. *D*, concentration-dependent binding of Eu³⁺-labeled vimentin to immobilized wild-type and cysteine-free R5. Wild-type R5 in the absence (●) or presence (○) of 1 mM DTT and cysteine-free R5 without DTT (▼) were coated onto microtiter plates at concentrations of 100 nm and overlaid with Eu³⁺-labeled recombinant vimentin at concentrations of 0.05-2 μM. Eu³⁺-Labeled vimentin bound to the different versions of R5 was measured as described under "Results." Scatchard plots of the binding data are shown.

formation between Cys1 and Cys4. The binding of vimentin to the BSA control or R4 was not observed (Fig. 4*C*, *lanes 1* and *2*).

The differential binding of vimentin to the reduced and oxidized forms of R5 prompted us to measure the binding affinities of R5 and its variants for vimentin. To this end, we titrated the binding of Eu³⁺-labeled vimentin to immobilized wild-type and cysteine-free mutant R5 proteins under reducing and nonreducing conditions. The dissociation constants obtained showed that wild-type R5 bound with 2-fold higher affinity to vimentin under reducing ($K_d = 0.155 \ \mu$ M) compared with nonreducing conditions $(K_d = 0.312 \ \mu \text{M})$, whereas an even higher binding affinity ($K_d = 0.096$ μ M) was observed for the mutant protein (Fig. 4D). Evidently, these differences in binding affinity reflect distinct structures of the reduced and nonreduced forms of R5 on the one hand and of wild-type R5 and its cysteine-free mutant on the other.

Nitrosylation of R5 Cys4 in Vitro—S-Nitrosylation is a reversible post-translational modification with a potential role in the regulation of protein function in response to oxidative stress. Mechanistically, S-nitrosylation is the reversible covalent binding of NO to a sulfhydryl group of a reactive cysteine, and

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whereas no plectin remained unbound at a ratio of 5:1 or 10:1 (Fig. 4*A*), indicating optimal binding at molar ratios somewhere in between 1:1 and 5:1. No significant differences in the vimentin binding abilities of wild-type R5 and its cysteine-free variant were detectable, however, using this assay. Regarding the controls, BSA and plectin R4 did not co-sediment with vimentin (Fig. 4*A*, *lanes 1* and 2), and when vimentin, BSA, both versions of R5, and R4 were incubated and processed alone, only polymerized vimentin was found in the pellets (Fig. 4*B*), except for a minor apparently self-aggregating fraction of Cys-free R5 (Fig. 4, compare *B*, *lane 4*, and *A*, *lane 4P*).

Next, we used a solid-phase binding assay in which soluble vimentin was overlaid onto nitrocellulose-immobilized plectin proteins in their reduced or oxidized forms. These experiments revealed that vimentin bound strongly to the reduced form (\sim 41 kDa) of monomeric wild-type R5 and its disulfide-linked (oxidized) higher order oligomers (Fig. 4*C*, *lanes 3* and 4), as well as to Cys-free R5 (*lane 5*). In contrast, vimentin showed no binding to the oxidized monomeric form (\sim 38 kDa) of wild-type R5 (Fig. 4*C*, *lane 3*). This suggested that the IF-binding site of plectin was not accessible for vimentin upon disulfide bond

it is precisely targeted to residues flanked by basic and acidic amino acids (28). As Cys4 in R5 meets these criteria, an *in vitro S*-nitrosylation assay was carried out using wild-type R5 and R5-C4S as the protein substrates, SNAP and *S*-nitrosoglutathione as the NO donors, and the biotin-switch method for detection of *S*-nitrosylated cysteines. In this assay, after blocking non-nitrosylated free thiol groups by methylation, *S*-nitrosylated cysteines are selectively identified by the cleavage of *S*-NO by ascorbate, followed by biotinylation of the free thiols, pull down of biotinylated proteins with streptavidin beads, and immunoblotting of eluates. As shown in Fig. 5*A*, we observed extensive nitrosylation of wild-type R5 preincubated with SNAP, whereas hardly any signal was detected with R5-C4S. Similar results were obtained with *S*-nitrosoglutathione as the NO donor (data not shown).

Nitrosylation of Plectin in Endothelial Cell Cultures—Nitric oxide plays a key regulatory role in endothelial cell function (29). To investigate whether plectin *S*-nitrosylation occurs *in vivo*, we incubated immortalized mouse renal endothelial cells in culture with SNAP and assayed plectin *S*-nitrosylation by the biotin-switch assay. As shown in Fig. 5*B*, a strong signal corre-



FIGURE 5. **Nitrosylation of R5 Cys4** *in vitro* and of plectin in cultured endothelial cells *in vivo. A*, nitrosylation *in vitro*. Purified wild-type (*wt*) plectin R5 and mutant R5-C4S proteins (80 μ g) were incubated with 100 μ M SNAP, and *S*-nitrosylated proteins were detected by the biotin-switch method. Biotinylated proteins were purified on streptavidin beads, eluted with β -mercaptoethanol, immunoblotted (SDS-10% PAGE), and probed with antibodies to the His tag. *B*, NO donor-mediated *S*-nitrosylation of plectin in cultured mouse renal endothelial cells. Cells preincubated with 100 μ M SNAP were lysed in blocking buffer (see "Experimental Procedures") and processed as described for *A*. *Upper panels*, eluted proteins were subjected to immunoblotting (SDS-5% PAGE) and probed with antibodies to plectin. Ascorbate (*Control*) was added prior to blocking free thiols. *Lower panel*, membranes were stripped and reblotted with anti-endothelial NOS (*eNOS*) antibody. *C*, *S*-nitrosylation of plectin in cultured mouse renal endothelial cells after stimulation of plectin in cultured motion of endogenous epithelial NOS. Cells were left untreated or were incubated with 100 nm PMA for 20 h, lysed, and processed as described for *B*. In *A*, 0.8% of the estarting material and 25% of the eluates were loaded onto the gels; in *B* and *C*, 3.3% of the cell lysates and 50% of the eluates were loaded.

sponding to full-length plectin could be detected in the streptavidin beads eluate, indicating S-nitrosylation of endogenous endothelial cell plectin. When ascorbate (1 mM) was added to the samples prior to blocking free (non-nitrosylated) thiol groups, no plectin signal was detectable in the eluate (Fig. 5B, Control), validating the assay. As an additional control, the membrane was stripped and overlaid with antibodies to endothelial NOS, which itself is a target of nitrosylation (30), revealing, as expected, the presence of the enzyme in both the starting cell lysate and the eluate recovered from the streptavidin beads (Fig. 5B, eNOS). We next examined whether plectin can be S-nitrosylated by an endogenous mechanism of NO generation. For this purpose, we treated cultured endothelial cells with PMA and subjected the cell lysates to the biotin-switch assay. PMA, an agonist of protein kinase C, has been shown to increase the expression and enzymatic activity of endothelial NOS in endothelial cells (31, 32). As shown in Fig. 5C, plectin was indeed S-nitrosylated by endogenously generated NO, whereas at basal NO levels (no PMA treatment), neither plectin nor endothelial NOS was nitrosylated.

S-Nitrosylation-induced IF Collapse Proceeds Faster in the Absence of Plectin—To assess whether S-nitrosylation has any effects on cellular IF cytoarchitecture, subconfluent cultures of immortalized mouse renal endothelial cells were subjected to immunofluorescence microscopy after prior exposure to the NO donor SNAP for 2, 4, or 6 h. Visualizing vimentin, the major constituent protein of endothelial IF networks, well spread cellular networks were observed at all time points, with no detectable differences becoming apparent between untreated and 6-h treated cells (Fig. 6, compare A-D). In contrast, when a similar experiment was carried out with immortalized plectin-deficient endothelial cells, a progressive collapse of vimentin filament networks into perinuclear bundles was observed. A partial collapse was visible in some cells already at the 2-h time point (Fig. 6G), whereas after 6 h, hardly any of the cells contained intact filaments (Fig. 61). In contrast, microtubules appeared to be unaffected by SNAP treatment (Fig. 6, *E* and *J*). A statistical evaluation of >100 wild-type and mutant cells each

per time point revealed that, whereas the proportions of intact (normal), partially collapsed, and fully collapsed IF networks were \sim 97:3:0 and \sim 95:4:1 in untreated and 6-h SNAP-exposed wild-type cells, respectively, the corresponding values were \sim 93:6:1 and \sim 1:8:91 in plectin-deficient cells (Fig. 6, *K*-*N*). This strongly suggested an antagonistic role of plectin in *S*-nitrosylation-mediated vimentin filament collapse.

DISCUSSION

Structural Implications of R5 Cysteines—The CD and fluorescence spectroscopy results suggested that wild-type R5 in the oxidized form is more compact (higher

 α -helical content) and more stable (higher resistance to urea denaturation) than the mutant without any cysteines, which resembles wild-type R5 in the reduced form. Thus, we infer that the formation of disulfide bridges could indeed contribute to the stabilization of the protein. Furthermore, our data revealed that R5 cysteines can form intramolecular disulfide bridges between Cys1 and Cys4 and that this oxidized R5 species migrates faster than its reduced counterpart, suggesting conformational alterations of R5 upon oxidation. As the modeling of the R5 module revealed Cys1 to be partially buried in the core of the protein, the presence of the linker in the cross-linked R5 species probably caused a change in the local conformation, exposing the surface in the vicinity of Cys1 and promoting the formation of a disulfide bridge.

Choi et al. (11) proposed that, in desmoplakin, each R domain represents an independent structure, with the three of them arranged as "beads-on-a-string." In a model proposed by Janda et al. (10) for the six R domains of plectin, the repeats are packed against each other in a way in which plectin R1-5 are arranged around a central R6 domain, forming a compact C-terminal globular or disk-like domain. It was suggested that, in addition to reciprocal hydrogen bond interactions mediated by hydrophobic residues on the surface of the repeats, linked cysteine residues could contribute to stabilizing this structure (10). Evidence in support of a compact packing of plectin R domains comes from electron microscopy, where the C-terminal domain was visualized as a globule 9 nm in diameter (9). Homology modeling of each of the six modules of plectin based on the known desmoplakin structure (50-73% sequence identity) yielded a cylinder-like configuration with an average size of 45×25 Å. Accommodation of six repeats within a 9-nm globule would indeed require tight packing of the repeats rather than a loose beads-on-a-string arrangement. R4 has a single cysteine in a position equivalent to Cys2 of R5; this cysteine can form disulfide bridges with cysteines of R5, linking these repeats together (Fig. 3B). We presume that corresponding cysteines in the other repeats could behave similarly. These find-



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ings lend further support to the model advanced by Janda *et al.* (10).

Comparison of Vimentin Binding of Plectin and Desmoplakin-In this study, the results of two experimental approaches, vimentin co-sedimentation and dot-blot overlay assays, clearly showed that R4 does not bind to vimentin, whereas R5 does. Furthermore, R4 did not co-localize with vimentin when overexpressed in PtK2 cells, nor did it cause the collapse of vimentin filaments (data not shown). This behavior is in full agreement with the results of a previous study in which the plectin vimentin-binding site was mapped to the linker region of R5 (14). IF binding of the corresponding linker regions has been shown for other plakin protein family members, such as envoplakin and periplakin (22). It should be noted, however, that an additional vimentin-binding site of plectin has been localized in its N-terminal actin-binding domain (33). On the other hand, Choi et al. (11) reported that each of the R domains of desmoplakin was able to bind to vimentin independently of the linker region. However, binding was very weak and occurred only if the R domains were present at a large molar excess, with a cooperative effect when all three repeats were present. There are two possible explanations for this apparent discrepancy. One is based on the assumption that proteins in the family are not redundant, but that each has a unique function. Thus, despite the overwhelming sequence similarity, desmoplakin could bind to vimentin in a different fashion or with different efficiency compared with plectin. In fact, plectin seems to have a higher affinity for vimentin than does desmoplakin, as concluded from the observation that plectin R5 cosedimented with vimentin at a molar ratio of 1:1. The other explanation could be the different conditions under which the assays were performed. As we have shown, single repeats of plectin bind to vimentin only in their reduced state. However, it appears that oxidation of the preparations of desmoplakin single repeats was not prevented (11), and as a consequence, binding was probably not optimal.

Functional Implications of R5 Cysteines and Plectin Nitrosylation—The binding of plectin to IFs is critical not only for the mechanical stabilization of cells, as plectin links the IF network to its anchorage sites at the plasma membrane, but also for a proper IF cytoarchitecture, with important consequences for signaling cascades controlling basic cellular functions (3-5). In this work, we have provided evidence that plectin R5 in its reduced form binds more efficiently to vimentin and has a more relaxed structure compared with its oxidized form. The dissociation constant of the cysteine-free R5 mutant (0.96×10^{-7} M) was 2- and 3-fold lower than those of the reduced and oxidized forms of R5, respectively. The substitution of a cysteine with a serine being limited to the exchange of an -SH group with an -OH group is a quite conservative mutation, which is not expected to cause a conformational change by itself. This could be the case, however, if the substitution disrupts the formation

Cysteines Proximal to the Plectin IF-binding Site

of one or more disulfide bridges. Because we have shown that Cys1 and Cys4 readily form a disulfide bridge that alters the conformational parameters of the repeat and that, when this happens, the tryptophan in the vicinity of Cys4 is buried in the structure, the finding that the cysteine-free mutant and the reduced form of R5 bind better to vimentin could be interpreted as a signal that, when the molecule is in a more open conformation, the vimentin-binding site might be more exposed, facilitating its interaction. In contrast, in the oxidized state, R5 appears to adopt a conformation in which the vimentin-binding site is inaccessible. A plectin molecule unable to bind to vimentin could severely affect cellular functions known to be IF-dependent, including structural reinforcement of the cytoplasm and control of signaling cascades, similar to the plectin-null situation (3-5). In the cellular context in which the protein is expressed in full length, Cys4 probably will not bind to Cys1, but might be linked to a cysteine in a different repeat or interacting protein. Thus, reversible in vivo modifications of Cys4 by local redox changes could lead either to cysteine oxidation through reactive oxygen species or to S-nitrosylation through reactive NO. In addition, reactive oxygen species could convert NO into higher oxides of nitrogen and radicals, such as peroxynitrite, which efficiently oxidize thiol groups (34).

Our study has shown shows that Cys4 in R5 is highly reactive and that plectin is a target for nitrosylation *in vitro* and *in vivo*. S-Nitrosylation of cysteines leading to nitrosothiols is a biochemical post-translational modification of proteins that allows cells to respond to environmental changes in specific subcellular compartments and can modulate protein activity in a fashion analogous to phosphorylation (34). Despite the existence of cysteine residues in virtually all proteins, proteomic analysis of S-nitrosylated proteins in extracts from brain (35), mesangial cells (36), and intact endothelial cells (37) revealed that *S*-nitrosylation is restricted to a small number of proteins. Furthermore, S-nitrosylation is not a function of total cysteine content because, generally, only one single cysteine thiol in the protein is modified. The specificity is determined by structural motifs around the cysteine and the intracellular proximity to a NO generator, such as the enzyme NOS, which produces NO through the conversion of L-arginine into L-citrulline. Both requirements seem to be fulfilled by Cys4 in R5. First, the residues upstream (Gln-Glu) and downstream (Glu) of Cys4 perfectly match the consensus motif for S-nitrosylation. Second, although a comprehensive co-localization study of endogenous NOS and plectin in different cell types and tissues has not been reported to date, at least for muscle, there is strong evidence for intracellular proximity. The majority of NOS activity in skeletal muscle is associated with neuronal NOS, one of three isoenzymes differentially expressed in different cell types. Being targeted to α_1 -syntrophin, a dystrophin-associated protein, neuronal NOS is found at specialized structures on the surface membrane of muscle fibers, such as neuromuscular and myo-

FIGURE 6. Immunofluorescence microscopy of wild-type and plectin-deficient endothelial cells after nitrosylation with SNAP. A–J, cells were left untreated or were treated with SNAP for 2, 4, or 6 prior to immunostaining using antibody to vimentin or tubulin as indicated. Scale bars = 30 μ m (A–E and J) and 20 μ m (F–H and J). K–N, shown is the statistical evaluation of cells with normal, partially collapsed, and completely collapsed vimentin networks. Data represent the means ± S.E. of three independent experiments. (More than 100 cells per experiment were counted from randomly chosen optical fields.) ple, plectin.

tendinous junctions and costameres (38). As a prominent component of neuromuscular and myotendinous junctions and costameres, plectin is also associated with the sarcolemma and has been found to co-localize with dystrophin (39, 40). Furthermore, there is evidence that plectin directly interacts with dystrophin and the dystrophin-associated transmembrane laminin receptor β -dystroglycan.⁴

Role for Plectin R5 Cys4 in NO-induced IF Collapse?—Assessing whether S-nitrosylation stimulated in endothelial cells by exposure to NO donors has any effects on cellular cytoarchitecture, we found an interesting link between plectin deficiency and dynamic properties of the IF network, which is built up of vimentin in these cells. Whereas well spread cellular vimentin networks largely persisted in cells treated with the NO donor SNAP for up to 6 h in wild-type cells, we observed a progressive collapse of vimentin filament networks into perinuclear bundles in plectin-deficient cells. One explanation for this phenomenon could be that plectin protects vimentin against oxidation. Like all other type III IFs, vimentin contains a single cysteine residue at a highly conserved position (position 328 in the human protein). Several reports suggest that this residue is oxidized in vivo. In fact, upon exposure of fibroblast-like synoviocytes to H₂O₂, it is oxidized in preference to other cytoskeleton proteins (41). Furthermore, these cells show an increased susceptibility to vimentin collapse around the nucleus upon exposure to oxidative stress, and vimentin IFs in rheumatoid synoviocytes are more susceptible than those in normal synoviocytes, bearing a possible pathological significance (42). The proposal that synoviocyte vimentin becomes glutathionylated (41) has been confirmed recently by proteomics approaches showing that vimentin is glutathionylated in human T lymphocytes and rat aortic smooth muscle cells (43, 44). Furthermore, prostanylation of the vimentin thiol has been shown in mesangial cells (45) and its nitrosylation in endothelial cells (46).

By binding to vimentin, plectin may either sterically hinder access of oxidants and thus prevent or impede IF collapse or prevent vimentin molecules from assuming a confirmation enabling vimentin-vimentin disulfide bridge formation. There is evidence, at least from *in vitro* experiments, that disulfidecross-linked vimentin homodimers or desmin/vimentin heterodimers are filament assembly-incompetent under nonreducing conditions (47–49). The proposed shielding effect of plectin on the vimentin thiol group may be further enhanced by the multitude of proteins bound to its surface as a consequence of its scaffolding function (3).

Alternatively, transient nitrosylation of Cys4 in R5 of plectin may promote its subsequent disulfide bond formation with the unique cysteine of vimentin, thereby preventing its bond formation with another vimentin molecule and eventually filament collapse. In fact, preliminary results indicate that R5 Cys4 of plectin can form disulfide bridges with the unique cysteine of vimentin *in vitro*.⁴

Another conceivable way that plectin might affect NO-induced IF network collapse involves protein phosphorylation. The reversible phosphorylation/dephosphorylation of IF sub-

⁴ R. Spurny, G. Rezniczek, M. Gregor, and G. Wiche, unpublished data.

unit proteins has been well established as a cellular mechanism leading to the disassembly of IFs. Recently, we found that the collapse of keratin networks induced in keratinocytes by the protein phosphatase inhibitor okadaic acid proceeds considerably faster in plectin-deficient compared with wild-type cells (5) and that the same applies to the vimentin IF network of fibroblasts.⁴ Several groups have reported inhibition of protein phosphatases by endogenously produced reactive oxygen species, and it has been shown that exposure of purified protein phosphatases to NO donors leads to reversible enzyme inhibition (50-52). Furthermore, NO activates serine/threonine kinases, including cGMP-activated protein kinase, as well as various tyrosine kinases (53, 54). Thus, the NO-triggered accelerated collapse of the vimentin network in plectin-deficient endothelial cells, similar to okadaic acid-treated cells, could be caused by protein hyperphosphorylation effected by NO-activated phosphorylation. The mechanism by which plectin counteracts/antagonizes hyperphosphorylation of vimentin networks in wild-type endothelial cells remains to be shown. However, one can expect that, similar to other cell types, plectin influences the cytoarchitecture of the IF network and thereby exerts regulatory functions over certain signaling cascades (3, 5). The collapse of the vimentin filament network probably compromises the normal ability of endothelial cells to respond to an appropriate stimulus, causing vasodilation in smooth muscle. As such a dysfunction is thought to be a key event in the development of atherosclerosis and has also been shown to be of prognostic significance in predicting vascular events, including stroke and heart attacks (55), it will be of interest to investigate whether plectin integrity plays a role in these diseases. In conjunction with plectin knock-out mouse models, the immortalized plectin-deficient endothelial cell line described here should be useful in delineating such relations as well as providing new perspectives on cytolinker protein functions.

In conclusion, we have shown that cysteines proximal to the IF-binding site of plectin in R5 have the capability to form disulfide bridges with consequences on the conformational and biochemical properties of the repeat domain. The redox state of these cysteines was found to have an influence on vimentin binding affinity, and one of the cysteines was identified as a specific target for nitrosylation. The observation that plectin-deficient endothelial cells were more sensitive to NO donor-induced IF network collapse compared with wildtype cells suggests an antagonistic role of plectin in oxidative stress-mediated alterations of the cytoskeleton and a possible role of a plectin cysteine as a regulatory switch.

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The Journal of Biological Chemistry

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